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Isolation and identification of a novel coronavirus from wild bird*

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Abstract A novel coronavirus strain was isolated from laryngotracheal swab of wild partridge and designated as partridge/GD/S14/2003 (S14). Its whole genomic sequence was obtained (GenBank Accession number: AY646283) through RT-PCR amplification, cloning, nucleotide sequencing, and analysis by the DNASTAR program. To investigate the origin of the virus, we further analyzed the nucleotide sequences of the main structural proteins, and compared those with other available virus isolates. Our results showed that the highest nucleotide homologies between the S1 gene of S14 strain and those of nephrogenic-type strains JX1-99 and TJ2-96 were 94.6% and 93.4%, respectively. In addition, a relatively high genetic identity, 85% and 84.3%, respectively, was detected between S1 gene of S14 and those of strains QXIBV and LX4. The results suggested that the S14 strain may be originated from or related to nephrogenic-type and proventriculus-type infectious bronchitis virus (IBV). The highest nucleotide homology between the S2 gene of S14 strain and those of QXIBV and LX4 was 85% and 84.3%, respectively and all of them belonged to group II coronaviruses. The highest nucleotide homology between the M gene of S14 strain and those of strains SAIB20 and GD6-98 was 90.6% and 90.2%, respectively by which S14 belonged to group III. Although they displayed high level of genetic identity in S1 and S2 gene, there was lower homology of M coding sequences between S14 and BJ, and between S14 and QXIBV strains. Phylogenetic analysis of N gene indicated that group I strains might evolve from RNA recombination between strain H52 and Gray; while group II strains from strain H120 and D1466. S14 strain had the highest N gene homology with strain QXIBV which was 95.7%, thus classified as a group III member. Strains SAIB20 and GD6-98 which were closely related to the M gene of S14 strain belonged to group I and IV, respectively. A possible role of partridge S14 strain may play in the process of coronavirus evolution is discussed.

Keywords: wild bird, coronavirus, structural protein genes, virus evolution.

Coronavirus exists widely in various kinds of domestic and wild animals. They often result in respiratory or intestinal illness including vomit, diarrhea and even death of the infected animals[1]. In this study, one wildbird coronavirus was isolated and characterized from a total of 1510 laryngotracheal swabs and 83 tissue samples, which were collected from wild poultry distributed in various areas of Guangdong Province, China. This strain was closely related to avian infectious bronchitis virus but distantly to SARS-CoV by phylogenetic comparison with viruses using genomic sequence data available in GenBank™.

1 Materials and methods

1.1 Materials

Viruses: Partridge coronavirus (designated as S14) was isolated and identified by the Key Laboratory of Poultry Feeding and Diseases Control, Ministry of Agriculture, China.

Reference strains: Respiratory-type strains included M41 (S1 M21883, S2: X04722, M: AF286184, N: M28566), Ark99 (S: L10384, N: M85244), SD1-97 (AY325732), DE072 (M: AF202999, N: AF203001), D1466 (S: M21971, M: AF203004, N: AF203006), HaN1-95 (S1: AY251817, M: AY325734), HaN2-95 (AY325728), GX1-98 (S1: AY319302, M: AF325727, N: AY278110), GX2-98 (S1: AY251816, M: AY325733, N: AY251816), H120 (S: M21970, M: AY028295, N: AY028296), H52 (S: AF352315, M: AF286185). CU-T2 (S: U04739, M: U46035, N: U04803) was a respiratory mutant type; nephrogenic-type strains included JX1-99 (AF210735), SAIBwj (AF397528), Holte (S: L18988; AF334685), Gray (S1: L14069, S2: AF394180), KB8523 (M21515), X (S1: AF427819, N: AY043315), BJ (S: AY3196551). Proventriculus-type strains included Q1 (AF286302), J2 (AF352312), T3 (AF227438),

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QXIBV (AF193423), LX4 (S: AY185197, N: AY338732), ZJ971 (S:AF352311, N:AF352308).

Specific-pathogen-free (SPF) embryonated chicken eggs: Nine-day-old SPF embryonated chicken eggs were kindly provided by Experimental Station of Veterinary Medicine, South China Agricultural University.

Total RNA Extraction Kit III was purchased from Sino-American Biotechnology Co. AMV XL, DNasin, 3′-Full RACE Core Set, 5′-Full RACE Core Set, pd(N)6, and Ex *Taq* enzyme were provided by TaKaRa Biotechnology (Dalian) Co., Ltd.

1.2 Methods

1.2.1 Virus propagation and RNA extraction S14 strain virus was inoculated to 10-day-old SPF embryonated chicken eggs, followed by incubation at 37℃ for 48 hours. Chicken embryos that died in 24 hours post-inoculation were discarded. Allantoic fluids were collected aseptically, centrifuged at 4000 rpm/min at 4℃, and then virus genomic RNA was extracted from the supernatant according to the protocols of the Total RNA Extraction Kit III (Sino-American Biotechology Ltd.).

1.2.2 RT-PCR amplification, cloning and sequencing of virus genome segments RT-PCR amplification and cloning of virus genome segments were carried out according to the methods of Ren et al.[6]. The extracted viral RNA was desolved in 11.5 μL DEPC-treated water and then reversely transcribed. The 3′- and 5′-Full RACE were performed according to the recommendations of the manufacturer (Takara). Positive clones were sent to Shanghai BioAsia Biological Technology Co., Ltd. for nucleotide sequencing.

A total of 29 pairs of primers (Table 1) were designed for cloning and sequencing of genomic segments and synthesized by Shanghai BioAsia Biological Technology CO., Ltd.

Table 1. Primer pairs designed

	Forward	Reverse	bp
1	5′-CAA GCC TAA AAC AGG GAG TAT C-3′	5′-GTT AAG TCA TTT CGC ATG CC -3′	940
2	5′-GGC ATG CGA AAT GAC TTA AC-3	5′-AGG TCG TCA CAC ATT TTC TC-3′	941
3	5′-GAG AAA ATG TGT GAC GAC CT -3′	5′-CAG AAC TCT TTA AAC TCA TCC A-3′	1421
4	5′-TGG ATG AGT TTA AAG AGT TCT G-3′	5′-GAG CGG TAT TTA ACA CCA T-3′	988
5	5′-ATG GTG TTA AAT ACC GCT C-3′	5′-AGG TTT ATT AAG GTG TTC TG-3′	800
6	5′-GCA GAA CAC CTT AAT AAA CCT-3′	5′-TTG CGC ATA ATA GAT GGC-3′	677
7	5′-GCC ATC TAT TAT GCG CAA -3′	5′-TTT CTC TAT TAA ACC AAG TAG GA-3′	1034
8	5′-TCC TAC TTG GTT TAA TAG AGA AA-3′	5′-ATT ACC AAC AAA CTC ATT GCC-3′	962
9	5′-GGC AAT GAG TTT GTT GGT AAT -3′	5′-TCA GTT CCA GTG TGT AAT GCA -3′	926
10	5′-TGC ATT ACA CAC TGG AAC TGA-3′	5′-TTC AAC ATT AAG CTT AGT CAA A-3′	1150
11	5′-TTT GAC TAA gCT TAA TGT TGA A -3′	5′-TCC AAA CAA CTG TTG AAT ATG -3′	1730
12	5′-CAT ATT CAA CAG TTG TTT GGA-3′	5′-GGT CTT TYT CCG TAG TAG GTA TT-3′	1628
13	5′-AAT ACC TAC TAC GGA RAA AGA CC-3′	5′-TAC TCT TGA TCC CAG TAC TTA AAG-3′	741
14	5′-CTT TAA GTA CTG GGA TCA AGA GTA-3′	5′-GGR TAG CCW GCA CTC TTA TC-3′	1324
15	5′-GAT AAG AGT GCW GGC TAY CC-3′	5′-ACT AGC ATT GTR TGT TGW GAA CA-3′	453
16	5′-TGT TCW CAA CAY ACA ATG CTA GT-3′	5′-CCA AAA ACR GTA CCA TTA GAT AC-3′	696
17	5′-GTA TCT AAT GGT ACY GTT TTT GG-3′	5′-TTT ATC TTA CCA TTA ATA AAK GAC-3′	1298
18	5′-GTC MTT TAT TAA TGG TAA GAT AAA-3′	5′-GTT GTA GCT CTA KAA CCA CAA GA-3′	481
19	5′-GTT CTT GTG GTT MTA GAG CTA CAA C-3′	5′-TTT GGC AGT GTG CGA ATR TT-3′	1326
20	5′-AAY ATT CGC ACA CTG CCA AA-3′	5′-TGT CTT TTG TTC AGT TTT CAA-3′	871
21	5′-TTG AAA ACT GAA CAA AAG ACA-3′	5′-CAT AAC TAA CAT AAG GGC AAT-3′	1372
22	5′-CAG TTT GTA GTT TCT GGT GG-3′	5′-TCA TTC CAC CAT TTW GAC AA-3′	840
23	5′-TTG TCW AAA TGG TGG AAT GA-3′	5′-ATT CCA ACC GTT CTT AGG-3′	657
24	5′-CCT AAG AAC GGT TGG AAT-3′	5′-TAC TCT CTA CAC ACA CAC -3′	632
25	5′-TGA CCA ARG CGG AAA TAA GA-3′	5′-AAT GAA RTC CCA ACG GAA AT-3′	1290
26	5′-GAT GGT ATA GTG TGG GTT-3′	5′-CTC CTC ATT CAT CTT GTC -3′	1644
27	5′-GAT GAC AAG ATG AAT GAG GA-3′	5′-AAG CTA CAT GCC TAT CTT CC-3′	1010
28	5′RACE1-F5′-CAT TTA GAA GAC ATC TTT GGT GTC-3′	5′ RACE1-R5′-TGT GAC GTA TAG AAA AAC AAA GCG TCA C-3′	604
29	3′RACE-F5′-GGA AGA TAG GCA TGT AGC TT -3′	3′RACE-R5′-CTG ATC TAG AGG TAC CGG ATC C-3′	332

Analysis of nucleotide sequence was carried out by the BLAST program and compared with published relevant sequences available in GenBank™. A number of representative viruses from different geographical locations were selected and analyzed by DNASTAR software package.

2 Results

2.1 RT-PCR amplification of S14 genome

S14 strain was used for RNA purification and RT-PCR amplification as described above. As shown in Fig. 1, we successfully obtained the cDNA fragments by genomic terminal RNA RACE (lane 1, 29), or routine RT-PCR.

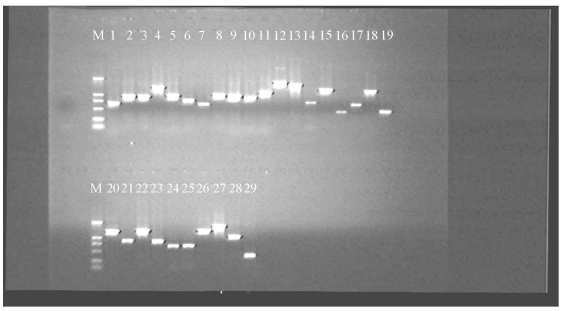

Fig. 1. RT-PCR amplification of S14 whole genomic RNA. M, Marker-DL2000; lane 1, 5′ RACE; lanes 2—28, cloning segments using the primer pairs as shown in Table 1; lane 29, 3′ RACE.

2.2 Analysis of nucleotide sequences of S1 protein genes and deduced amino acid sequence

S1 hypervariable regions of the two strains (M41 and S14) were obtained by sequencing, which included the nucleotides from start coden ATG to the cleavage site of S precursor protein. S1 gene of S14 strain was 1620 bp long, encoding 540 amino acids and containing 19 potential N-glycosylation sites. The G+C percentage was 35.49%. Hydrophilic analysis of deduced amino acid sequences of S1 protein showed that the first 20 N-terminal amino acid residues of S1 protein of S14 virus formed a hydrophobic region, which was presumed as the signal sequence of S precursor protein. Amino acid sequences at the cleavage site of S precursor protein were RRFRR.

Compared with reference strain M41, there were several insertions and deletions in S14 strain: a TCT insertion at position 67, ATTA at 216, ATAATCCA at 245, A at 353, G at 266, A at 350, AG at 360, TCT at 362, ATGG at 420 and CT at 429. On the other hand, the deletions of ATTCATGGTGGT at position 221—233, T at 236, A at 262, ATGGCC at 415—421 were identified. There were similar mutations in LX4 and QXIBV strains, inserting TC and AG at position 360, respectively. There were also some nucleotide substitutions in S14 strain. Compared with position 25 of M41 strain, the deduced amino acid sequence of S14 had an Asn (N) insertion at this point and Gly (G) + Ser (S) at position 119.

Fig. 2 shows four groups of infectious bronchitis viruses (IBV). Group I could be divided into two subgroups, in which Gray, Vic S, JMK strains belong to subgroup A and others belong to subgroup B. Group II could also be divided into two subgroups, with S14, JX1-99, and TJ2-96 strains classified to subgroup C and other strains to subgroup D. Subgroup B strains had high genetic identity with strains H52, H120 and M41. It was speculated that Group I viruses were mutated from vaccine virus. All Group II viruses were isolates from China including published nephrogenic-type and proventriculus-type strains. S14 strain was also found in this group. Gx2-98 isolate from China of Group III had close identity with strain Holte, and it was deduced that it might be mutated from Holte. Group IV strains were proventriculus-type infectious bronchitis viruses isolated in China, the S1 gene of which was distantly related to those of other viruses.

2.3 Analysis of nucleotide sequences of S2 gene and deduced amino acid sequence

The coding region of S2 protein of the two strains (M41 and S14) was obtained by nucleotide sequencing analysis, covering the nucleotides from the cleavage site of S precursor protein to the stop codon. S2 protein coding region of S14 strain was 1878 bp in length, which encodes 625 amino acids. The percentage of G+C was 35.52%. S14 strain had 13 N-glycosylation sites through analysis of glycosylation recognition signals. There was a hydrophobic region at position 550 to 590 in the C-terminal, which might be the connecting region of envelope and nucleocapsid. Compared with the published sequence of M41 strain (GenBank accession number: M21883), there were many nucleotide mutations in S2 protein gene of S14 strain.

Coronavirus strains of Group I were closely related to H52, M41 and Holte. All Group II viruses, including published nephrogenic-type and proventriculus-type strains, were isolated from China. S14 strain

was also in this group. Taken together, there was close genetic identity between Group I strains and Group II strains, while Group III viruses were representatives for American respiratory-type and vaccine strains (Fig. 3).

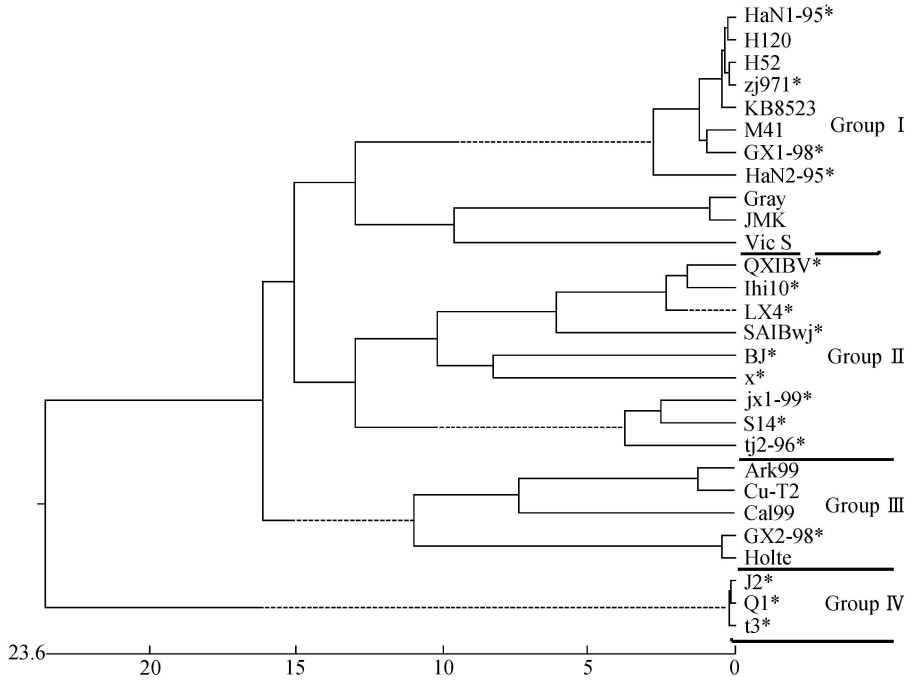

Fig. 2. Phylogenetic tree of S1 gene. * IBV isolates from China.

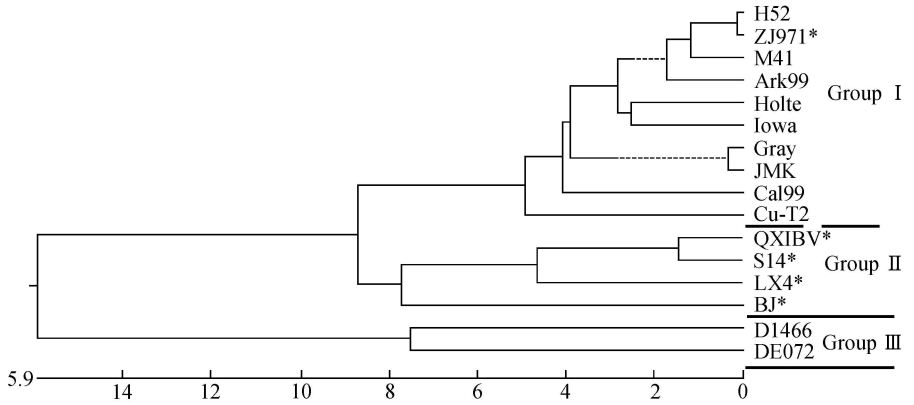

Fig. 3. Phylogenetic tree of S2 protein gene. * IBV isolates in China.

2.4 Analysis of nucleotide sequences of M protein gene and deduced amino acid sequence

Nucleotide sequences and deduced amino acid sequence of M protein of these two strains were further analyzed. The open reading frame of the M protein of S14 strain was 678 bp in length, encoding 225 amino acids. There were two N-glycosylation sites at the N-terminal of S14 strain. The first 11 amino acids of S14 strain were hydrophilic and were presumed as the outmembrane domain of M protein, their downstream 20 to 100 amino acids formed three hydrophobic regions and were considered as three transmembrane domains.

In comparison with the published sequence of H52-GD (GenBank accession number: AY044184), there were many nucleotide substitutions but no insertion or deletion in M protein gene of S14 strain.

Based on M gene dendrogram, Group I could be further divided into subgroups A and B. While JL1-97, GX1-98, DE072, HaN2-95, and HEN4-94 were closely related to H52, M41 belonged to subgroup A. D1466, H120, SAIBwj, SD1-97, SC2-93 and

Cal99, which had the highest genetic dentity with H120, belonged to subgroup B. S14 belonged to group III, and was also closely related to Groups I and II but distantly to Group IV (Fig. 4).

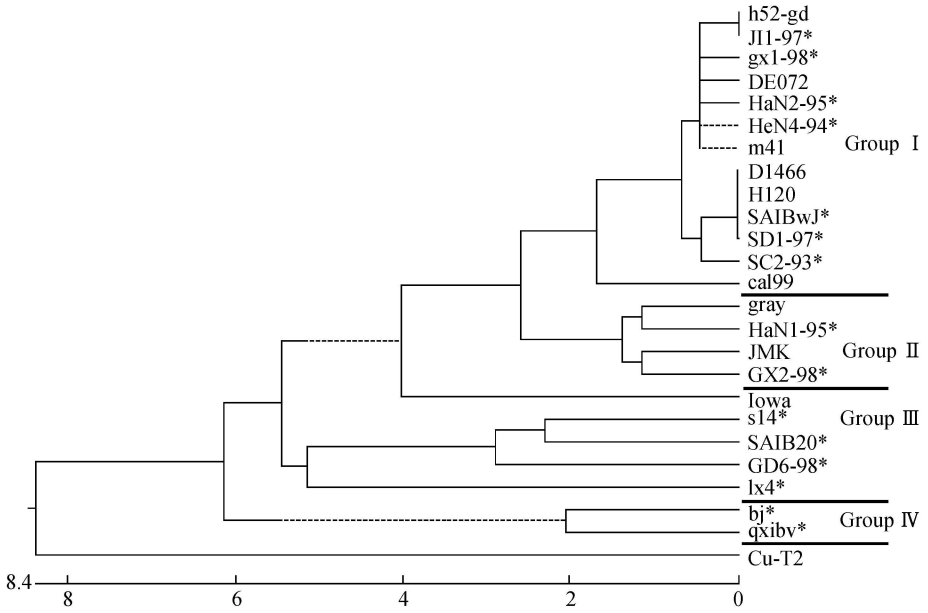

Fig. 4. Phylogenetic tree of M protein gene. * IBV isolates in China

2.5 Analysis of nucleotide sequences of N protein gene and deduced amino acid sequence

Nucleotide sequence and deduced amino acid sequence of N protein gene of two strains, M14 and S14, were obtained by sequencing. The open reading frame of N protein of S14 strain was 1230 bp in length, encoding 409 amino acids. There was one N-glycosylation site at the N-terminal of S14 strain, with another in the middle. N protein was hydrophilic, which may be beneficial for the interaction of N protein and RNA.

In comparison with the published sequence of M41 (GenBank accession number: M28566), the N protein gene of S14 strain had nucleotide substitutions but no insertion or deletion.

Based on the analysis of N protein gene, Group I could be further divided into subgroups A and B, of which JL1-97, SAIB14 and SAIBwj closely related to H52 belonged to subgroup A and SAIB20, GX1-98, GX2-98 which had the highest identity with Gray and Ark99 belonged to subgroup B. Group II could also be divided into C and D subgroups, of which SC2-93, SD1-97 closely related to D1466 belonged to subgroup C and HaN2-95, GD1-87 which had the highest identity with H120 belonged to subgroup D. S14 strain had the highest identity with QXIBV and belonged to Group III (Fig. 5).

3 Discussion

As an RNA virus, IBV is prone to genetic mutation and recombination during the process of replication, resulting in the emergence of new mutant strains with different serotypes. Therefore, it is difficult to develop effective vaccines against IBV infection. As a result, avian infectious bronchitis still poses significant economic threat to the poultry industry. In our previous work, we obtained four coronavirus isolates from wild partridge, one of which is designated as Partridge coronavirus S14.

Phylogenitic analysis of the whole genome of S14 helped us find out the origin of the virus. By analyzing the structural protein genes (S1, S2, M and N) of S14, S14 was clustered with Group Ⅱ viruses by S1 gene, the viruses in this group are all nephrogenic-type and proventriculus-type IBVs isolated from China. S14 had a high identity with nephrogenic-type strains JX1-99, TJ2-96, QXIBV, LX4 and BJ. While S2 gene of S14 strain had a much higher genetic identity with the Chinese proventriculus-type strains QXIBV and LX4, which was 97.2% and 90.6%, respectively. They formed a subgroup II with another Chinese IBV isolate BJ. Phylogenetic analysis of M gene showed that S14 belonged to Group III and had a higher genetic identity with the Chinese isolates SAIB20, GD6-98 and LX4 than that

of BJ and QXIBV. Analysis of N gene grouped S14 to Group III which included the Chinese IBV strains QXIBV, LX4 and BJ.

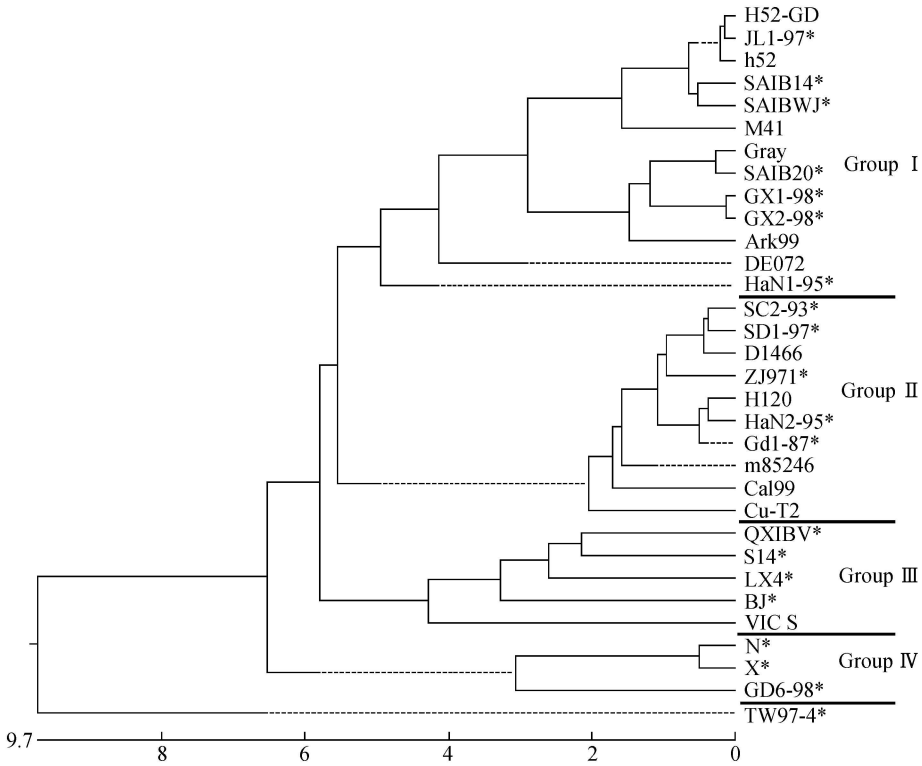

Fig. 5. Phylogenetic tree of N protein gene. * IBV isolates in China.

It was considered that S2 and N genes of IBV were conserved. In our research, the S2 and N genes of S14 were closely related to the Chinese IBV isolates such as QXIBV, LX4 and BJ, so was the S1 gene. However, it was not the case for M gene. Analysis of M gene showed that the M gene of S14 was closely related to Iowa, SAIB20, GD6-98 and LX4 but very distantly to BJ and QXIBV. Since S1, S2 and N genes of S14 were closely related to those of Chinese isolates QXIBV and BJ, whose sequences were all distantly related to those of strains from other countries, QXIBV and BJ might be the virulent isolates in China where S14 was originated. As far as M gene was concerned, S14 was not closely related to vaccine strains H52 and H120 which were widely used in China. Therefore, it was predicted that the evolution of S14 was also related to the mutation of vaccine strains. Some new IBV strains in China and other countries might be the recombinants of vaccine strains and field strains[6,7]. Further study should be carried out to determine whether S14 was the recombinant of IBV isolates in China and introduced vaccine strains.

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